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Minireview

MINIREVIEW

Looking beyond *Saccharomyces*: the potential of non-conventional yeast species for desirable traits in bioethanol fermentation

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One sentence summary: Some non-conventional yeast species have excellent stress tolerance characteristics for industrial ethanol fermentations.
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ABSTRACT

Saccharomyces cerevisiae has been used for millennia in the production of food and beverages and is by far the most studied yeast species. Currently, it is also the most used microorganism in the production of first-generation bioethanol from sugar or starch crops. Second-generation bioethanol, on the other hand, is produced from lignocellulosic feedstocks that are pretreated and hydrolyzed to obtain monomeric sugars, mainly D-glucose, D-xylose and L-arabinose. Recently, *S. cerevisiae* recombinant strains capable of fermenting pentose sugars have been generated. However, the pretreatment of the biomass results in hydrolysates with high osmolarity and high concentrations of inhibitors. These compounds negatively influence the fermentation process. Therefore, robust strains with high stress tolerance are required. Up to now, more than 2000 yeast species have been described and some of these could provide a solution to these limitations because of their high tolerance to the most predominant stress conditions present in a second-generation bioethanol reactor. In this review, we will summarize what is known about the non-conventional yeast species showing unusual tolerance to these stresses, namely *Zygosaccharomyces rouxii* (osmotolerance), *Kluyveromyces marxianus* and *Ogataea (Hansenula) polymorpha* (thermotolerance), *Dekkera bruxellensis* (ethanol tolerance), *Pichia kudriavzevii* (furan derivatives tolerance) and *Z. bailii* (acetic acid tolerance).

Keywords: yeasts; non-*Saccharomyces*; phenotype; stress tolerance; bioethanol

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INTRODUCTION

Yeasts are one of the best-studied microbial groups in nature. More than a thousand unique yeast species have been described in the literature (Boekhout 2005). Many of these species have been associated with human activity, such as the production of fermented beverages, for thousands of years (Sicard and Legras 2011). Taxonomic analyses of the microbiota present in spontaneous alcoholic fermentation processes revealed huge yeast diversity with one yeast species dominating most of the fermentation processes, namely the ascomycetous yeast *Saccharomyces cerevisiae* (Pretorius 2000; Pando Bedriñana, Querol Simón and Suárez Valles 2010; Meersman *et al.* 2013; Bokulich *et al.* 2014; Steensels and Verstrepen 2014). This species became the model organism for eukaryotic cell research, providing countless data and allowing enormous expansion of scientific knowledge (Botstein, Chervitz and Cherry 1997). It was the first eukaryotic microbial species of which the whole genome was sequenced (Goffeau *et al.* 1996). The millennia-long evolution conferred *S. cerevisiae* with the ability to proliferate both in aerobic and anaerobic conditions and to accumulate high concentrations of ethanol, which makes it an obvious choice as starter culture for food and beverage fermentations (Querol 2003). However, more recent industrial applications, such as the production of bioethanol, confronts *S. cerevisiae* with new, very specific challenges that differ from those encountered in many food fermentations. They include different environmental stresses and tolerance against cytotoxic inhibitory compounds (Palmqvist and Hahn-Hägerdal 2000; Almeida, Modig and Petersson 2007; Basso, Basso and Rocha 2011; Taylor *et al.* 2012). On top of that, there is strong pressure to improve the economic viability of second generation bioethanol production and this is bringing us to the limits of what *S. cerevisiae* can offer in terms of fermentation performance in lignocellulose hydrolysates. This motivated researchers to explore alternatives beyond the conventional *Saccharomyces* species.

Non-conventional yeasts present a huge, yet barely exploited, resource of yeast biodiversity. Many of these non-conventional yeast species exhibit industrially relevant traits such as the ability to utilize complex substrates as nutrients, extreme tolerance against stress and fermentation inhibitors. They developed specific mechanisms to survive under extreme environmental conditions. The evolution of most of these species was independent of that of *S. cerevisiae* (Souciet *et al.* 2009) and therefore, it is widely speculated that most of these species possess novel and unique mechanisms that are not present in the model yeast. To date, most of them have been characterized as spoilage yeast due to their frequent isolation from contaminated foods and beverages (Kubaczka and Ge 1999; Martorell *et al.* 2007; Dujon 2010). However, the next-generation sequencing technology and the advanced molecular engineering tools offer the possibility to reveal the underlying molecular basis of the superior stress tolerance of these non-conventional yeast species. In this review, we describe the phenotypic landscape of some of these non-conventional yeasts that are extremely tolerant to stresses commonly encountered during first- and second-generation bioethanol production, namely osmotic stress, ethanol stress, thermal stress and different fermentation inhibitor stresses. Additionally, we also discussed the available tools for genetic modification of these species. Together, our review provides an overview of the potential industrial application of these non-conventional yeast species.

OSMOTOLERANCE

Yeast cells are exposed to osmotic stress during industrial fermentation. Especially, the implementation of very high gravity (VHG) fermentation with initial sugar concentrations above 300 g L^{-1} necessitates the introduction of osmotolerant yeasts (Watanabe *et al.* 2010; Puligundla *et al.* 2011; Tao *et al.* 2012; Pais *et al.* 2013). Therefore, yeast's ability to sustain growth in high sugar or salt environments has been a trait of interest for decades. Molecular mechanisms responsible for osmotolerance in *S. cerevisiae* have been broadly described in previous studies (Mager and Varela 1993; Albertyn *et al.* 1994; Garay-Arroyo and Covarrubias 1999; Davis 2000; Hohmann 2002; Erasmus, Vandermerwe and Vanvuuren 2003; Wojda 2003). While *S. cerevisiae* has remained the model organism to investigate the molecular basis of this trait, researchers identified extremely osmotolerant non-conventional yeasts, such as *Zygosaccharomyces rouxii* (Kubaczka and Ge 1999; Kinclová, Potier and Sychrová 2001; Martorell *et al.* 2007; Leandro *et al.* 2011). Below we describe the physiological characteristics and the industrial potential of *Z. rouxii*.

Zygosaccharomyces rouxii

Zygosaccharomyces rouxii is a haploid yeast belonging to the hemiascomycetous yeast phylum. It is recognized as one of the most osmotolerant and halotoreant species, being able to grow up to 90% (w/v) of sugar concentrations (Martorell *et al.* 2007), in contrast to *S. cerevisae* that remains viable only up to 50% (w/v) of sugar (Restaino *et al.* 1983; Mukherjee *et al.* 2014), and able to grow in up to 3 M NaCl (Iwaki *et al.* 1998), whereas halotolerant mutants of *S. cerevisiae* survive only up to 2 M NaCl (Gaxiola, Corona and Zinker 1996). *Zygosaccharomyces rouxii* has been described as a spoilage yeast because of its frequent isolation from contaminated sugar- or salt-rich foods and beverages (Restaino *et al.* 1983; Martorell *et al.* 2007). Nevertheless, it has also been used for vinegar and soy sauce production, where it plays an important role in flavor formation (Hamada *et al.* 1991). Researchers identified a close phylogenetic relationship between *S. cerevisiae* and *Z. rouxii*. The divergence of this two species has occurred nearly 100 million years ago prior to the whole genome duplication event (Souciet *et al.* 2009; Dashko *et al.* 2014).

Substantial efforts have been made to develop molecular tools for *Z. rouxii* genome modification (Table 1) (Pribylova and Sychrova 2003; Pribylova, de Montigny and Sychrova 2007). In addition to that, the Génolevures Consortium made public the sequence of *Z. rouxii* CBS 732 type strain (Souciet *et al.* 2009), consisting of seven chromosomes with a total size of 9.8 Mb. This solved the controversy concerning the number of chromosomes and the genome size of this strain, either seven chromosomes with a total size of 12.8 Mb (Sychrova *et al.* 2000) or six chromosomes with 12.7 Mb (Solieri *et al.* 2008). However, sequencing data obtained from several other *Z. rouxii* isolates suggests high genomic diversity among the strains of this species (Souciet *et al.* 2009).

Using *S. cerevisiae* as model yeast, it has been established that plasma membrane transport systems are involved in the response to elevated salt conditions. Moreover, it has been found that all yeast species with plasma membrane antiporters possess at least one antiporter with broad specificity for both Na^+ and K^+ (or their analogues Li^+ and Rb^+) (Potier, Sychrova and Kinclova 2001; Papouskova and Sychrova 2006; Velkova and Sychrova 2006). Initially, *Z. rouxii* was considered to be an exception and was believed to possess only one type of plasma membrane antiporter, ZrSod2–22p, capable of Na^+ (Li^+) extrusion.

Table 1. Overview of available genetic tools and summary of stress resistance of all discussed species.

Species	Glucose	Salt	Temp	Ethanol	5-HMF	Acetic acid	Tools for genetic manipulation	Sequenced strains and accession number
Zygosaccharomyces rouxii	90%(w/v)	3M NaCl					(a) Efficient homologous recombination (b) Modified yeast-based plasmids	CBS 732 - NCBI 012990
Kluyveromyces marxianus			52°C				Non-Homologous End Joining-mediated integrative transformation	DMB1- NCBI BBIL00000000 CBS 6556
Ogataea polymorpha			50°C				(a) Expression vectors with different inducible and constitutive promoters (b) Large rage of selectable markers (c) Non-homologous and homologous recombination	CBS4732 (CCY38–22–2; ATCC34438, NRRL-Y-5445), NCYC495 (CBS1976; ATAA14754, NRLL-Y-1798) and DL-1 (NRRL-Y-7560; ATCC26012
Dekkera bruxellensis			>35°C	10–16%			Modified transformation protocols	AWRI1499-AHIQ0100000 CBS2499-NCBI SRR065689
Issatchenkia orientalis	48% (w/v)	0,85M NaCl	<45°C		7 g L^{-1}		–	M12- ALNQ00000000
Zygosaccharomyces bailii	60% (w/v)		40°C			24 g L^{-1}	Homologous recombination	CBS 680 - ISA1307

However, Sychrova *et al.* (2000) refuted this assumption and reported that the isolation of ZrNha1, which based on sequence homology with *S. cerevisae* Nha1, could represent a *Z. rouxii* antiporter with broad substrate specificity (Pribylova, Papouskova and Sychrova 2008). Experimental data have shown that ZrNha1 is indeed capable of efficient K^+ and moderate Na^+ transport, thus playing a more complex role in *Z. rouxii* physiology. Moreover, ZrSod2–22 antiporter was confirmed to only transport Na^+, which indicates that it plays a role in cell detoxification. Based on their observations, Pribylova, Papouskova and Sychrova (2008) concluded that *S. cerevisae* and *Z. rouxii* have different strategies to deal with salt stress.

Investigations to identify the mechanisms behind the extraordinary resistance to high sugar concentrations of *Z. rouxii* have resulted in the identification of two plasma membrane sugar transporters: ZrFfz1 and ZrFfz2. Interestingly, they are displaying different substrate preference, as ZrFfz1 is showing high specificity for fructose transport, while ZrFfz2 is mostly involved in glucose transport. Moreover, both ZrFfz1 and ZrFfz2 are phylogenetically distant from other known fungal sugar transporters, which might be one of the reasons for the unique resistance to high sugar conditions (Leandro *et al.* 2011).

Moreover, *Z. rouxii* can survive a wide range of pH at a high glucose concentration (Tokuoka 1993). However, low pH resistance combined with high glucose concentration is dependent on the pH-reducing agent. For instance, *Z. rouxii* is more sensitive to citric acid, commonly used as food preservative, than to HCl (pH 3–5 vs pH 1.5, respectively) (Restaino *et al.* 1983; Tokuoka 1993; Membré, Kubaczka and Chéné 1999). Interestingly, when 0.5% acetic acid was combined with a high salt concentration (18%), the growth of *Z. rouxii* was significantly inhibited, probably due to the reduction of proton expulsive activity (Kusumegi, Yoshida and Tomiyama 1998).

The unusual phenotype of *Z. rouxii* makes it an interesting candidate for industrial application, such as already done for soy sauce fermentation (Schoondermark-stolk *et al.* 2002). It is also used as dry starter for miso fermentation (Sujaya *et al.* 2003).

THERMOTOLERANCE

Thermotolerance is a highly desirable trait for fermenting microorganisms used in fuel ethanol production. Efficient bioethanol production requires high-temperature conditions (approximately 50°C) for the enzymatic saccharification of the

biomass prior to fermentation (Tabka *et al.* 2006). Moreover, high-temperature fermentation lowers cooling costs, particularly in tropical countries where average day-time temperatures are usually high throughout the year (Anderson, Mcneil and Watson 1986). It is also believed that high-temperature fermentation conditions decrease the risk of contamination. *Saccharomyces cerevisiae* is the most broadly used microorganism in current fuel ethanol production processes. However, its limited temperature tolerance (optimum range for fermentation: 25–37°C) increases the cost of ethanol production (Nonklang *et al.* 2008; Abdel-Banat *et al.* 2010). In order to reach efficient fermentation in high-temperature conditions, it is necessary to obtain a thermotolerant microorganism that cannot only survive elevated temperatures but also produces efficiently ethanol at high temperature (Limtong, Sringiew and Yongmanitchai 2007). This explains the high interest in understanding the molecular mechanisms enabling certain non-conventional yeast species of producing and accumulating ethanol under high thermal stress.

Kluyveromyces marxianus

Kluyveromyces marxianus is well known for its extreme thermotolerance. It has been reported to grow at 47°C (Limtong, Sringiew and Yongmanitchai 2007), 49°C and even up to 52°C (Banat, Nigam and Marchant 1992) and to produce ethanol at temperatures above 40° (Kourkoutas *et al.* 2002; Limtong, Sringiew and Yongmanitchai 2007; Nonklang *et al.* 2008). *Kluyveromyces marxianus* is not only thermotolerant but also offers additional benefits including a high growth rate and the ability to utilize a wide variety of industrially relevant substrates such as sugar cane, corn silage juice, molasses and whey powder. It has also been used for recombinant protein (Nonklang *et al.* 2008) and industrial enzyme production, such as inulinase (Rouwenhorst *et al.* 1988) and β-galactosidase (Martins *et al.* 2002).

Kluyveromyces marxianus was first described in 1888 by E.C. Hansen, being named *S. marxianus*. Numerous strains have been isolated since, mostly from cheese and other dairy products. Strains of the *Kluyveromyces* genus have the ability to mate and produce fertile hybrids, both intraspecies and interspecies hybrids. Frequent isolation of these hybrids leads to difficulties in identifying a distinct species. This problem has been addressed by DNA reassociation studies (Fuson, Presley and Phaff 1987; Martini *et al.* 1987; Llorente 2000). *Kluyveromyces marxianus* shows a high intraspecies polymorphism with a common species-specific pattern (Belloch *et al.* 1998). The strain *K. marxianus* CBS 6556 (KCTC 17555 = ATCC 26548) has been sequenced and a genome size of 10.9 Mb has been estimated (Jeong *et al.* 2012).

The metabolism of *K. marxianus* has been described as respirofermentative. Interestingly, *K. marxianus* along with its sister species *K. lactis* is traditionally classified as 'Crabtree negative' yeasts. It has been suggested that such a contradiction might be due to highly divergent phenotypes among isolates (Lane *et al.* 2011). In contrast to *S. cerevisiae*, *K. marxianus* is able to utilize xylose, xylitol, cellobiose, lactose and arabinose both on solid and liquid media (Nonklang *et al.* 2008). It has a demonstrated ability to ferment glucose between 30 and 45°C. At 30°C, it achieved similar levels of ethanol yield and glucose consumption as *S. cerevisiae* while at 45°C, *S. cerevisiae* was unable to ferment (Nonklang *et al.* 2008). Previous reports described the temperature tolerance range of several *K. marxianus* isolates, with most able to grow at 42°C and only few up to 48°C (Nonklang *et al.* 2008; Abdel-Banat *et al.* 2010; Lane *et al.* 2011; Hu *et al.* 2012) The mechanisms behind this extreme temperature tolerance are unknown up to date. In the last decade, the availability of tools for genetic modification of *K. marxianus* is increasing (Table 1) (Kegel *et al.* 2006; Pecota, Rajgarhia and Da Silva 2007; Nonklang *et al.* 2008; Abdel-Banat *et al.* 2010; Lee *et al.* 2013; Yarimizu *et al.* 2013; Hoshida *et al.* 2014), which will bring new opportunities to uncover the molecular basic of this unique phenotype.

Ogataea polymorpha

Ogataea polymorpha (syn. *Hansenula polymorpha* and *Pichia angusta*) together with *K. marxianus* one of the two yeast species having strains is able to grow at temperatures higher than 50°C (Shin, Hong and Bae 1996; Péter *et al.* 2007). It is also one of the few yeast species designated as methylotrophic since it can utilize methanol as sole carbon and energy source (Ogata, Nishikawa and Ohsugi 1969; Kurtzman 2011; Yurimoto, Oku and Sakai 2011).

The mechanism behind this high-temperature tolerance is not yet elucidated. It is known that, similarly to other fungi, *O. polymorpha* accumulates trehalose and expresses heat shock proteins (Hsps) under heat shock conditions (Reinders *et al.* 1999; Guerra *et al.* 2005), although this response is suppressed under hypoxia unlike *S. cerevisiae*. After heat shock, *O. polymorpha* experiences a cell cycle arrest that is longer than in *S. cerevisiae* (Guerra *et al.* 2005). Moreover, in contrast to *S. cerevisiae*, *TPS1* gene deletion, which encodes the initial enzyme of trehalose synthesis, does not cause lack of growth in glucose-containing media. However, *tps1*Δ mutants are more sensitive to heat shock than wild-type strains (Reinders *et al.* 1999).

Because of its temperature tolerance and its ability to ferment xylose and cellobiose to ethanol, *O. polymorpha* has been suggested as a potential microorganism to be used in simultaneous saccharification and fermentation (SSF) (Ryabova, Chmil and Sibirny 2003). Nevertheless, the ethanol yield is not high enough for economic feasibility. In order to address this problem, a combination of metabolic engineering and classical selection is currently used to improve ethanol production of *O. polymorpha* (Ishchuk *et al.* 2008, 2009; Kurylenko *et al.* 2014). The best-performing strain accumulated 10 g L^{-1} of ethanol at 45°C in semi-aerobic conditions (Kurylenko *et al.* 2014).

Currently, *O. polymorpha* is a preferred host for recombinant protein production since it has strong methanol-inducible promoters (Hartner and Glieder 2006), secretes proteins efficiently (van Dijk *et al.* 2000) and produces less hyperglycosylated proteins compared to *S. cerevisiae* (Kim *et al.* 2013). In addition to its application in industry, *O. polymorpha* is also used as a model system in fundamental research; especially in the study of methanol metabolism (van Zutphen *et al.* 2010), peroxisome biogenesis and degradation (Veenhuis *et al.* 1992), and nitrate transport and assimilation (Avila *et al.* 1995; Siverio 2002).

Because of its relevance in the industry and in basic research, genetic tools have been developed (Table 1) (Saraya *et al.* 2012). Several strains of this species have been sequenced (Table 1) (Ramezani-Rad *et al.* 2003; Ravin *et al.* 2013). The genome of the strain DL-1 showed about 10% sequence divergence to the other two strains (Hanson, Byrne and Wolfe 2014). This difference in sequence supports the previous reclassification of the DL-1 in a separate species named *O. parapolymorpha* (James and Stratford 2011).

ETHANOL TOLERANCE

Saccharomyces cerevisiae has been well described as the most ethanol-tolerant yeast species. Approximately 100 million

years ago, rapid redesigning of the carbon metabolism pathway allowed *S. cerevisiae* lineage to suppress respiratory metabolism and, thereby, accumulate ethanol. Most of the non-conventional yeast species did not undergo these molecular events along their course of evolution and they lack efficient fermentation performance under industrial conditions. However, several individual studies identified strains of non-conventional yeast species, such as *Dekkera bruxellensis, Pichia kudriavzevii, Schizo saccharomycespombe, Torulaspora delbrueckii* and *Wickerhamomyces anomala*, with promising fermentative features and similar ethanol tolerance levels as those of *S. cerevisiae* (Galafassi *et al.* 2011; Zha *et al.* 2013; Mukherjee *et al.* 2014; Ruyters *et al.* 2015). Among these species, *D. bruxellensis* has been described as one of the most promising alternative yeasts in terms of ethanol tolerance and production. Both *S. cerevisiae* and *D. bruxellensis* share at least some molecular features responsible for this trait: duplication of its alcohol dehydrogenase-encoding ADH genes (reviewed in Piskur and Langkjaer 2004; Piškur *et al.* 2006) and promoter rewiring where the cis-regulatory motif (AATTTT) was absent in the respiration-associated genes and present at the conserved position in the rapid growth-associated genes (Rozpędowska *et al.* 2011). Some essential characteristics of *D. bruxellensis* that could be mined for industrial application are discussed below.

Dekkera bruxellensis

Dekkera (anomorph *Brettanomyces*) yeasts are often isolated from similar niches as *Saccharomyces* yeasts e.g. beer, wine and cider and are generally considered as spoilage yeasts due to their contribution towards increasing phenolic off flavors in beer and wine (Piškur *et al.* 2012; Echeverrigaray *et al.* 2013). However, secondary fermentation with the same species brings a characteristic flavor profile for certain specialty beers, such as lambic beers (reviewed by Schifferdecker *et al.* 2014).

During the course of evolution, the *Dekkera* lineage separated from that of the *Saccharomyces* clade over 200 million years ago (Procházka *et al.* 2010; Rozpędowska *et al.* 2011). Nevertheless, the *Dekkera* clade demonstrates unusual resemblance with *S. cerevisiae* in terms of physiological traits. Interestingly, both are facultative anaerobic and crabtree positive, petite positive (i.e. able to produce offspring without mitochondrial DNA), tolerant to ethanol, able to produce and accumulate high levels of ethanol and able to grow in acidic environments (Rozpędowska *et al.* 2011; Piškur *et al.* 2012). These two lineages shared similar niches containing large amounts of sugars and finally acquired similar traits independently. Therefore, the study of Rozpędowska *et al.* (2011) proposed that a parallel evolution took place. Wijsman *et al.* (1984) reported a complex pattern of substrate consumption and metabolite production profile for *D. bruxellensis*. Firstly, glucose is dissimilated to ethanol and acetic acid, then most of the ethanol is oxidized to acetic acid and finally the acetic acid produced in the previous phases is converted to CO_2 and water during the lag phase.

The presence of *D. bruxellensis* in wine fermentation clearly indicates a high ethanol tolerance of this species (Renouf *et al.* 2006; Piškur *et al.* 2012; Echeverrigaray *et al.* 2013). Strains isolated from wine fermentation showed ethanol tolerance between 10 and 16% (v/v) (Echeverrigaray *et al.* 2013). Moreover, several studies reported that the ethanol yield of *D. bruxellensis* in batch culture under anaerobic conditions is comparable to that of *S. cerevisiae* and its close relatives (Galafassi *et al.* 2011; Rozpędowska *et al.* 2011). *Dekkera bruxellensis* demonstrated an ability to 'compete' with *S. cerevisiae* and emerged as the dominant ethanol-producing microbe in industrial ethanol plants (de Souza Liberal *et al.* 2007; Passoth, Blomqvist and Schnürer 2007) presumably because of its advantage over *S. cerevisiae* in nitrate assimilation during industrial fermentation processes (de Barros Pita *et al.* 2011). Whole genome sequencing and analysis confirmed the enrichment in transporters, enzymes associated with nitrogen and lipid metabolism and along with oxidoreductase enzymes, which could explain its ability to survive in high ethanol environments as well as in anaerobic conditions when the regeneration of NAD(P)+ is impaired (Woolfit *et al.* 2007; Curtin *et al.* 2012).

Dekkera bruxellensis accumulates acetic acid, being reported as more acetic acid tolerant than *S. cerevisiae* (Rozpędowska *et al.* 2011). However, it is rather thermosensitive, with 30°C being its optimal temperature for biomass production and being thermosensitive already at 35°C (Brandam *et al.* 2008; Taillandier *et al.* 2014). In addition, Aguilar-Uscanga *et al.* (2011) investigated the effect of different initial glucose concentrations on growth and ethanol production and noticed that up to 93 g L^{-1} the growth and ethanol production rate remains optimal.

Unlike *S. cerevisiae*, genetic modification of *D. bruxellensis* is difficult. Making simple hybrids and performing basic transformation is still a major hurdle with this species because of its extremely complex genome. Major progress was reported by Miklenić *et al.* (2013). In this study, a modified LiAc/PEG electroporation method was used for transformation of *D. bruxellensis*, with a transformation efficiency ranging from 0.6 to 20 transformants/μg. In addition, the newest sequencing technology provides a robust platform to investigate further the genomic organization of this species and identify the causative genes or mutations that are responsible for superior traits. There is huge intraspecies karyotype polymorphism (from 4 to at least 9) and, moreover, it could not be described as haploid or diploid due to the high frequency of polymorphic sites (approximately 1%) in its genome (Hellborg and Piskur 2009). The same study also estimated the genome size of *D. bruxellensis* in the range of 20–30 Mb by pulsed-field electrophoresis of several European strains. However, the *de novo* assembly of the *D. bruxellensis* AWRI1499 genome under the assumption of a diploid strain yielded a 12.7 Mb assembly (Curtin *et al.* 2012). To date, also the strain CBS2499 has been sequenced and the genome is publicly available (Table 1) (Piškur *et al.* 2012). Furthermore, short read sequences of three other *D. bruxellensis* strains have recently become publicly available, namely VIB X9085 ST05.12/22 (Crauwels *et al.* 2014) and AWRI1608 and AWRI1613 (Borneman *et al.* 2014).

FURAN DERIVATIVE TOLERANCE

The hydrolysate obtained from second-generation biomass and used for bioethanol production is highly complex. A number of byproducts, cytotoxic to yeast, are released during pretreatment (reviewed in Taylor *et al.* 2012). The composition and concentration of these inhibitory compounds greatly vary depending on the nature of the feedstock and pretreatment method (Zha, Muilwijk and Coulier 2012). During pretreatment and enzymatic hydrolysis, the hemicellulose fraction of the biomass is decomposed into different hexose sugars such as D-glucose, D-galactose, D-mannose and D-rhamnose, as well as pentose sugars, including D-xylose and L-arabinose (reviewed by Palmqvist and Hahn-Hägerdal 2000; Almeida, Modig and Petersson 2007). The cellulose fraction hydrolyzes to glucose. At high temperature and pressure, hexose and pentose sugars are degraded to hydroxymethylfurfural (HMF) and furfural, respectively, due to dehydration (Jing and Lü 2008). HMF and

furfural are known to have damaging effects on RNA, DNA, proteins and membranes even at low concentrations (Janzowski *et al.* 2000; Lin, Qiao and Yuan 2009). Detoxification of these inhibitory compounds is highly expensive and, therefore, furan-tolerant yeast strains are more practical to improve industrial second-generation bioethanol fermentation performance. Researchers invested considerable efforts to reveal the molecular basis of superior HMF tolerance in *S. cerevisiae*. It has been suggested that at least three MAPK-signalling pathways have a role in mediating HMF tolerance in *S. cerevisiae*, especially the cell-wall integrity pathway, and the phosphatidylinositol signalling pathways (Zhou *et al.* 2014). Moreover, the disruption of *SIZ1*, a gene encoding an E3 SUMO-protein ligase, confers a significant increase in furfural tolerance in comparison to other previously reported metabolic engineering strategies in *S. cerevisiae* (Xiao and Zhao 2014). However, so far little has been revealed regarding the molecular basis of superior furan tolerance of certain non-conventional yeast species, namely *W. anomalus, P. kudriavzevii, Candida stellata, C. ethanolica, P. fermentans* and *Z. bailii* (Mukherjee *et al.*, unpublished observations). Among these species, *P. kudriavzevii* has been reported to withstand more than 7 g L^{-1} of 5-HMF (Ruyters *et al.* 2015) and is often studied for high-temperature industrial bioethanol fermentation (Dhaliwal *et al.* 2011; Kwon *et al.* 2011; Isono *et al.* 2012; Oberoi *et al.* 2012). Therefore, in this review we discuss the physiological characteristics and the industrially relevant properties of *P. kudriavzevii*.

Pichia kudriavzevii

Pichia kudriavzevii (syn *Issatchenkia orientalis*) has been isolated from a variety of niches, including sourdough (Meroth, Hammes and Hertel 2003), cocoa bean fermentation (Dandi *et al.* 2009), mango pulp peel compost (Dandi, Dandi and Chaudhari 2013), Champús-a Colombian cereal-based beverage (Osorio-Cadavid *et al.* 2008), fermented butter, Tanzanian fermented togwa, fermented pineapple juice (Chan *et al.* 2012), soil (Mukherjee *et al.* 2014), sugar cane juice (Dhaliwal *et al.* 2011; Oberoi *et al.* 2012), cornstalk, sweet sorghum stalk and rice straw (Kwon *et al.* 2011). This indicates the ability of *P. kudriavzevii* to grow on complex substrates. The study of Oberoi *et al.* (2012) confirmed that *P. kudriavzevii* can grow on glucose, sucrose, fructose and mannose but it only weakly assimilates galactose. However, it does not metabolize sugars like maltose, xylose, arabinose, cellobiose, raffinose or trehalose. A recent study described *P. kudriavzevii* as a crabtree-negative yeast species (Schnierda *et al.* 2014).

Several studies revealed the extremely robust physiology of *P. kudriavzevii*. Its tolerance to furan derivatives has been reported in two studies. The result of Kwon *et al.* (2011) showed that *P. kudriavzevii* is highly tolerant to up to 3 g L^{-1} furfural, it displays sensitivity from 5 g L^{-1} and gets completely inhibited at 7 g L^{-1}. The same study reported that it was able to tolerate up to 5 g L^{-1} of 5-HMF without any major growth inhibition but was completely inhibited by 7 g L^{-1} of 5-HMF. However, the study of Ruyters *et al.* (2015) showed that for certain strains the tolerance limit can exceed 7 g L^{-1} of 5-HMF. *Pichia kudriavzevii* is also tolerant to several other fermentation inhibitory compounds relevant to second-generation bioethanol production. For example, it tolerates concentrations of acetic acid of up to 8–10 g L^{-1} (Oberoi *et al.* 2012; Dandi, Dandi and Chaudhari 2013). It has also been reported to be tolerant to up to 2 g L^{-1} of formic acid (Dandi, Dandi and Chaudhari 2013) and between 1.8 and 2 g L^{-1} of vanillin (Kwon *et al.* 2011; Dandi, Dandi and Chaudhari 2013). This species has also been characterized for tolerance to several other environmental stress factors that are relevant to bioethanol production. For example, it has often been identified as a thermotolerant, ethanologenic yeast species (Dhaliwal *et al.* 2011; Kwon *et al.* 2011; Isono *et al.* 2012; Oberoi *et al.* 2012). It is more efficient than *S. cerevisiae* in ethanol production at temperatures higher than 35°C and can ferment at up to 45°C (Oberoi *et al.* 2012). *Pichia kudriavzevii* can grow at extremely low pH conditions (down to pH 2) (Daniel *et al.* 2009; Kitagawa and Tokuhiro 2010). Moreover, it achieves 20% more ethanol yield compared to *S. cerevisiae* under low pH conditions (pH 4) (Oberoi *et al.* 2012). The same study also identified the salt and sugar tolerance of this species and reported that *P. kudriavzevii* can tolerate 5% (w/v) of NaCl (0.85M) and 40% (w/v) of glucose. This agrees with the results of Ruyters *et al.* (2015), which confirmed the ability of a soil isolate of *P. kudriavzevii* to grow at 48% (w/v) of glucose. This species has also been evaluated for ethanol tolerance and can tolerate up to 12% (v/v) (Ruyters *et al.* 2015) or 15% (v/v) (Daniel *et al.* 2009) ethanol.

The tools and technologies for genome engineering of *P. kudriavzevii* are extremely limited. Only recently, the draft genome of *P. kudriavzevii* (M12 strain) has been determined and annotated in order to exploit the full potential of this multitolerant species by understanding the genetic organization and metabolic pathways (Chan *et al.* 2012). The genome sequence has been deposited at DDBJ/EMBL/GenBank (Table 1). To date, the only attempt of genome engineering was performed by Kitagawa *et al.* (2010). This study successfully constructed a β-glucosidase expression system in the *P. kudriavzevii* MF-121 strain for efficient conversion of cellobiose to ethanol.

ACETIC ACID TOLERANCE

Tolerance to weak acids is crucial for industrial yeast strains used in second-generation bioethanol production. During acid pretreatment of lignocellulose raw material, acetic acid is the most abundant weak acid generated, with a concentration ranging between 5 and 10 g L^{-1} (Martinez *et al.* 2001; Qian *et al.* 2006; Villarreal *et al.* 2006; Chandel *et al.* 2007). It is produced when the acetyl groups of the hemicellulose are released during pretreatment. Weak acids are known to have a cytotoxic effect. They enter the yeast cells in the non-dissociated form by passive diffusion through the plasma membrane and possibly also through the Fps1 aquaglyceroporin channel (Mollapour and Piper 2007). Once inside the cell, acetic acid dissociates into acetate and a proton due to the neutral pH of the cytoplasm. The protons accumulate and acidify the cytosol causing detrimental effects on cell metabolism (Arneborg, Jespersen and Jakobsen 2000; Brett *et al.* 2005), such as the inhibition of the glycolytic enzymes (Pampulha and Loureiro-Dias 1990) and the NADH dehydrogenase (Ding *et al.* 2009). Subsequently, the low intracellular pH inhibits yeast growth, prolongs lag phase and reduces ethanol production (Limtong *et al.* 2000; Cantarella *et al.* 2004). Therefore, the development of robust weak acid-tolerant strains is of primary importance for industrial bioethanol fermentations.

In the food industry, acetic acid is commonly used as a preservative since it inhibits the growth of yeasts and molds (Lambert and Stratford 1999). Nevertheless, there are yeasts that can grow in those harsh conditions. This is the case for *Z. bailli*, which is the most acetic acid-tolerant species currently described (Lindberg *et al.* 2013).

Zygosaccharomyces bailii

Zygosaccharomyces bailii is often reported in association with food spoilage due to its resistance to weak acid preservatives

(Martorell *et al.* 2007; James and Stratford 2011; Stratford *et al.* 2013) and ability to grow at pH 2 (Praphailong and Fleet 1997). In addition, it can adapt to high temperatures and sugar concentrations with maximum reported growth in 60% (w/v) glucose at 40°C (Pitt and Hocking 2009). This species demonstrates a much higher tolerance to acetic acid than *S. cerevisiae*. At a concentration of 24 g L^{-1} acetic acid, the reduction in μmax (maximum specific growth rate) in *Z. bailii* is comparable to the one in *S. cerevisiae* at a concentration of 9 g L^{-1} acetic acid (pH 5) (Lindberg *et al.* 2013).

Zygosaccharomyces bailii is a Crabtree-positive yeast and it is described as fructophilic (Pina *et al.* 2004). The efficiency of ethanol fermentation with *Z. bailii* under aerobic conditions is dependent on the available carbon source. In glucose cultures, ethanol is produced at a lower rate, while in fructose a higher rate and higher yield were reported (Merico *et al.* 2003). It can grow anaerobically in complex media but not in simple defined media, even when they are supplemented with unsaturated fatty acids and sterols (Rodrigues *et al.* 2001). The high tolerance of *Z. bailii* to acetic acid has been related to different mechanisms. One of them is the ability to simultaneously consume acetic acid and glucose (Sousa, Rodrigues and Crte-real 1998). On the contrary, active acetic acid transport is repressed by glucose in *S. cerevisiae* (Cassio, Leao and van Uden 1987). Nevertheless, some commercial *S. cerevisiae* wine strains are able to consume simultaneously both substrates (Vilela-Moura *et al.* 2008). Other mechanisms proposed are the ability of *Z. bailii* to maintain proper intracellular pH (Prudêncio, Sansonetty and Côrte-Real 1998) and to rearrange its lipid composition (Lindberg *et al.* 2013) in the presence of acetic acid. The basal level of complex sphingolipids was significantly higher in *Z. bailii* than in *S. cerevisiae*, further emphasizing the proposed link between lipid saturation, high sphingolipid levels and acetic acid tolerance.

Because of the properties described above, *Z. bailii* was suggested as a suitable host for the production of heterologous proteins and organic acids (Branduardi *et al.* 2004; Vigentini *et al.* 2005; Dato *et al.* 2010). Moreover, inulinases naturally produced by *Z. bailii* can hydrolyze inuline rich media resulting in glucose and fructose syrups, which can be further used in bioethanol production and biodesulfurization processes (Paixão *et al.* 2013).

Most of the genetic tools available for *S. cerevisiae* could be optimized for *Z. bailii* (Table 1) (Mollapour and Piper 2001; Branduardi, Dato and Porro 2014). Furthermore, *Z. bailii* is a diploid yeast (Mollapour and Piper 2001; Rodrigues *et al.* 2004) and one of the major challenges for its genetic modification is the impossibility to obtain stable *Z. bailii* haploid strains (Branduardi, Dato and Porro 2014). Recently, the strain CBS 680 (Galeote *et al.* 2013) and a hybrid strain (ISA1307) (Table 1) (Mira *et al.* 2014) have been sequenced. The neotype strain CBS 680 does not show some of the common traits of the species and, therefore, CBS 685 (NCYC 563) and NCYC 1766 have been suggested as better representatives (James and Stratford 2011).

CONCLUSIONS

Saccharomyces cerevisiae is one of the best known currently used industrial species. However, to be used in second-generation bioethanol production, it still has to overcome a variety of stresses present during the process that damage cell metabolism and consequently reduce the ethanol yield and the fermentation rate. Despite the efforts made in engineering *S. cerevisiae* to mitigate these detrimental effects, *S. cerevisiae* unfortunately still has its limitations. On the other hand, less studied yeasts, known as non-conventional yeast species, present better tolerance to some of these stresses and could potentially be used as model organisms to study the molecular basis of these tolerances in order to further develop *S. cerevisiae*. One of the examples is high osmotic stress, especially in VHG bioethanol fermentation. A species that can cope with this environment much better than *S. cerevisiae* is *Z. rouxii*, which is able to grow in salt concentrations of 3 M NaCl and sugar concentrations up to 90% due to its unique plasma membrane sugar transporters (Leandro *et al.* 2011). The other essential trait for efficient bioethanol production is the tolerance to temperatures of up to 50°C. This is important in SSF in which the enzymes active in the hydrolysis have an optimal temperature of 55°C (Olofsson, Bertilsson and Lidén 2008). This temperature is quite distant from the optimal fermentation temperature of *S. cerevisiae*, which ranges between 25°C and 37°C (Nonklang *et al.* 2008; Abdel-Banat *et al.* 2010). However, engineered strains from the species *K. marxianus* and *O. polymorpha* have been reported to ferment xylose at 45°C, but with an ethanol yield which is still far from industrially profitable (Kurylenko *et al.* 2014).

Presence of inhibitors such as acetic acid and furan derivatives in the second-generation bioethanol hydrolysate is another impediment towards efficient ethanol fermentation by *S. cerevisiae*. The yeast species so far described to be the most acetic acid tolerant is *Z. bailii*. It can grow at a concentration as high as 24 g L^{-1} while *S. cerevisiae* shows a comparable growth at a concentration of 9 g L^{-1} (Lindberg *et al.* 2013). Regarding furan derivatives resistance, *P. kudriavzevii* seems to be tolerant to concentrations of 5-HMF as high as 7 g L^{-1}. Finally, the tolerance to ethanol is a crucial limiting factor in bioethanol production. *Saccharomyces cerevisiae* seems to be the most resistant species to this stress but some research groups have reported that *D. bruxellensis* is also an ethanol-tolerant yeast since it can handle concentrations ranging from 10 from 16% (v/v). The genomes of all these non-conventional yeasts have been sequenced and for most of them a range of genetic tools is currently available. Nevertheless, the molecular mechanisms underlying the tolerance of these species to these stress conditions remain poorly investigated for all of them.

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